Retention of Insect Virus Infectivity in Mammalian Cell Cultures

Arthur H. McIntosh and Karl Maramorosch
Boyce Thompson Institute, Yonkers, New York 10701

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Abstract: The present and future use of polyhedral insect viruses for biological control justifies extensive tests for their safety, not only as possible disease agents of various hosts but also as possible inducers of other virus infections, transforming factors, and causes of allergies. This paper describes the retention of an insect polyhedral virus in cultured human cells. No deleterious effects of this virus on growing cells was observed. Polyhedral inclusion bodies (PIB) and free virions of the nuclear polyhedrosis virus (NPV) of *Heliothis zea* (cotton bollworm) were studied to determine their effects on several human cell cultures. No cytopathic effect (CPE) was observed in cultures of primary human amnion (PHA), foreskin (HF), embryo (EM), lung (WI38), and leucocytes (LEU) inoculated with both PIB and virions. Cell viability studies employing HF and LEU cultures revealed no difference between controls and cultures inoculated with PIB over a 3-week period. PHA and WI38 cultures inoculated with virions and stained with hematoxylin and eosin showed no presence of inclusion bodies or other cytopathic effects. However, virus was recovered from PHA, WI38, and LEU cultures by bioassay 4 weeks after inoculation. Immunofluorescence of PHA cultures inoculated with the NPV failed to detect antigen over a 5-week period. DNA synthesis of LEU cultures was unaffected by exposure to the virus as assessed by autoradiography, nor did such cultures ever transform. PHA cultures inoculated with NPV did not show the presence of any transformed foci over a 4-month period. A neutralization test whereby antiserum could be tested for its neutralizing capacity in a bioassay test was developed.

INTRODUCTION

Although the control of insect pests by viruses is by no means a recent innovation, this method is receiving ever-increasing attention. The reasons for this probably are the apparent high degree of specificity of insect viruses, leaving other species and predators unharmed, and a desire to decrease the use of chemical insecticides wherever possible. Since there have been relatively few reports (Himeno *et al.*, 1967; Ignoffo and Rafajko, 1972) concerning the effects of insect viruses on mammalian cell cultures, the present study was initiated employing a commercially available product, *Heliothis* (cotton bollworm) nuclear polyhedrosis virus (NPV).

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MATERIALS AND METHODS

Cell cultures. Human foreskin (HF) and embryonic (EM) cultures were obtained from the Cell Culture Division, Naval Biomedical Research Laboratory, Oakland, California. WI38 diploid (lung) cell line was purchased from the American Type Culture Collection (ATCC), Rockville, Maryland. Primary human amnion (PHA)[1] and leucocyte (LEU) cultures from normal individuals were prepared in this laboratory according to well-established techniques (Chang, 1968; Chang *et al.*, 1971). Cell cultures were grown in both roller tubes (15 × 150 mm) and Leighton tubes at 35 C. Roller-tube cultures were placed on a roller drum (15 rev/h) following inoculation with virus.

Nutrient media. All cultures with the exception of leucocytes were grown in Eagle's minimal essential medium (MEM) (Eagle, 1959), containing 10% inactivated fetal bovine serum and 1 ml of 200 mM glutamine per 100 ml medium. Leucocytes were cultured in medium 199 (Morgan *et al.*, 1950), containing 20% inactivated fetal bovine serum and glutamine as described. Antibiotics were incorporated into media at concentrations of 50 units/ml penicillin and 50 μg/ml streptomycin. In addition, amphotericin at 2.5 μg/ml was included in media when cultures were inoculated with crude polyhedral inclusion bodies (PIB).

Virus. Polyhedral inclusion bodies (PIB) of *Heliothis zea* (Cotton bollworm) nuclear polyhedrosis virus (NPV) and ova were generously donated by International Minerals and Chemical Corp., Libertyville, Illinois. The commercial PIB preparation known as Viron/H contains at least 4×10^9 PIB per gram of product. Crude PIB were prepared by suspending 0.25 g of Viron/H in 25 ml of Hanks' balanced salt solution (HBSS) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin. The suspension was allowed to stand for 10 min, centrifuged at 500 rpm for 5 min, and the supernatant removed and recentrifuged at 2,000 rpm for 30 min. The pellet was resuspended in HBSS, suitably diluted, and PIB counted in a hemocytometer. LEU, HF, EM, and PHA cultures were inoculated with 2.74×10^5 to 1.25×10^7 PIB/ml.

Virions were released from PIB by the following method: 0.6 g of Viron/H was resuspended in 2 ml of distilled water and layered onto a 40 to 65% (w/v) linear sucrose gradient. Preparations were centrifuged for 1 hr at 24,000 rpm in a Beckman L3–40 ultracentrifuge with an SW 27 rotor at 4 C. The resulting white band 5 to 6 cm from the bottom of the meniscus was removed by aspiration. PIB from several such runs were pooled together. Residual

[1] Full-term human placentas delivered by Caesarian section were obtained through the courtesy of St. John's Riverside Hospital, Yonkers, New York.

sucrose was removed by diluting with distilled water and centrifuging at 5,000 rpm for 1 h or by dialyzing against distilled water and concentration of PIB by centrifugation. 10^8 to 10^{10} PIB were treated with an equal volume of alkali (0.05 M Na_2CO_3 + 0.05 M NaCl, pH 9.5) for 30 to 60 min. The alkali digestion was arrested by careful neutralization with 0.2 N HCl, and the suspension centrifuged at 5,000 rpm for 1 h. Examination of supernatant fluids by electron microscopy revealed the presence of bacilliform particles typical of NPV. This virus suspension was used as a source of inoculum for cell cultures.

SV40 virus was obtained from the ATCC and passed three times in primary African green monkey kidney (PAGMK) cells. Pooled fluids and cells from the last passage were frozen and thawed several times to disrupt cells and then passed through a 0.45 μm (Millipore Corp.) filter. The virus pool titered 10^6 infectious units/ml in a established African green monkey kidney cell line (CV-1). For the determination of enhancing or inhibiting effect on SV40, PHA cultures were inoculated with 0.1 ml of *Heliothis* NPV (from the digestion of 10^{10} PIB) and 3 days later challenged with 0.1 ml of 10^5 infectious units of SV40. Controls consisted of PHA cultures challenged with SV40 alone and uninoculated PHA cultures.

Immunofluorescence. PHA cultures in Leighton tubes were inoculated with 0.1 ml of virus suspension derived from the digestion of 10^9 PIB. Cultures were incubated at 35 C for 2 h, the cell sheaths washed twice with HBSS, and fresh media added. Coverslips were removed at weekly intervals for 5 weeks and prepared for immunofluorescence by an indirect fluorescent antibody method previously described (McIntosh and Chang, 1971). The fluorescent conjugate employed was goat-anti rabbit globulin (Microbiological Associates Inc., Bethesda, Maryland). A Nikon microscope with fluorescent attachment was used to examine slides.

Immunization of rabbits. Female New Zealand white rabbits weighing 6 to 7 lb were prebled and immunized using a foot-pad technique similar to that described by Gray *et al.* (1966). The challenge dose consisted of virions released from the alkali digestion of 10^8 PIB. Rabbits were bled from the marginal ear veins employing a rabbit bleeding apparatus (Bellco, Vineland, New Jersey). All sera were stored at –20 C.

Autoradiography. Each leucocyte culture was inoculated with 0.1 ml of a virus suspension obtained from the alkali digestion of 10^8 PIB. Cultures were exposed to 1 μCi/ml of thymidine-methyl-^{3}H (specific activity 6.7 Ci/mM) 24 h prior to termination of the experiment on the seventh day. Controls consisted of uninoculated cultures treated in the same manner. For autoradiography, cultures were centrifuged at 1,000 rpm for 10 min in a clinical

centrifuge and the pellet washed several times with PBS. Smears from the cell suspensions were made on clean microscopic glass slides, air dried, and dipped in liquid emulsion as previously described (McIntosh and Chang, 1971). The percentage of cells showing nuclear labeling was scored in inoculated and control cultures.

Bioassay. PIB and virus containing suspensions from inoculated cultures were tested using a sensitive neonatal-larvae-feeding technique (Ignoffo, 1966).

Neutralization test. 0.6 ml of virus suspension (from the digestion of 10^9 PIB) was mixed with 0.6 ml of antiserum (appropriately diluted) and placed at 35 C for 2 h. At the end of this period, 0.1 ml aliquots were plated onto the surface of the diet in the neonatal bioassay test. Controls consisted of pre-antiserum plus virus and postantiserum plus PIB.

Viable cell counts. The method described by Merchant *et al.* (1964) employing 0.4% erythrosin B was employed.

RESULTS

Cytopathology. Of four cultures (PHA, HF, EM, and WI38) inoculated with PIB and free virions of *Heliothis*, none showed any CPE over a 4-week period. In addition, coverslips from PHA, HF, and WI38 inoculated cultures were removed at weekly intervals and stained with hematoxylin and eosin. No inclusion bodies were observed in any of the inoculated cultures with the exception of PHA. However, this property was nontransmissible and an extensive search of control cultures showed a low proportion of cells with such inclusions in the nuclei. Inoculated HF cells were passaged three times at weekly intervals without the appearance of any cytopathic effects.

Cell viability studies. HF cultures inoculated with 2.74×10^5 and 2.74×10^6 PIB per culture gave average cell viability figures of 77% and 84% (for two experiments), respectively, as compared with 94% for control cultures at 2 weeks. Similarly, EM cultures gave values of 67% and 85% as compared with 79% for controls under the above described conditions. In addition, LEU cultures inoculated with 1.25×10^7 PIB/culture gave an average cell viability count from two experiments of 4.50×10^5 cells/culture as compared with 2.65×10^5 cells/culture for controls.

DNA synthesis. Since it is known that some vertebrate viruses can cause an increase in DNA synthesis of leucocyte cultures (Brody *et al.*, 1968), this property was examined with the *Heliothis* NPV. The average percentage of cells showing nuclear labeling, from two experiments in which 2,000 cells were examined, were 4.00% and 1.98% for inoculated cultures and 1.33% and 1.39%, respectively, for controls.

Table 1. The effect of *Heliothis zea* nuclear polyhedrosis virus on the transformation of primary human amnion cultures by SV40

Experiment	Proportion of cultures transformed		
	Control	SV40	SV40-NPV
I	0/7	4/8	8/8
II	0/4	5/7	7/7
III	0/6	6/6	7/7

Transformation. Two approaches to the study of viral transformation were employed in this investigation. The first was the examination of inoculated PHA cultures for the presence of transformed foci. The second was the determination of cell proliferation in LEU cultures inoculated with the NPV. Both PHA and LEU cultures were kept for 4 to 6 months following inoculation. A total of eight experiments were performed. No development of foci in PHA cultures nor increase in cell numbers in LEU cultures was observed.

It has been reported that superinfecting viruses may be either enhanced or inhibited by other viruses (Tsuchiya and Rouhandeh, 1971). Table 1 illustrates the results of such a study where PHA cultures were inoculated with the NPV followed by superinfection with SV40. As can be seen, only in one experiment did there appear to be an enhancing effect. There was no difference in percent of cells showing presence of T antigen in SV40 and SV40-NPV inoculated cultures. SV40 virus was also recovered from both groups of transformed cultures.

Viral persistence in cell cultures. Table 2 illustrates the recovery of *H. zea* NPV from three inoculated cultures. With the exception of WI38, mortalities at the fourth-week test period were greater than 50%. The reason for the low

Table 2. The recovery of *Heliothis zea* nuclear polyhedrosis virus from inoculated cell cultures

Culture	% Mortality[a]					
	Time of assay[b] (days)					
	0	3	7	14	24	28
Virus	100	–	–	–	–	–
PHA	–	100	89	41	63	63
Control	–	4	–	–	–	–
WI38	–	100	88	71	45	5
Control	–	–	9	–	–	–
LEU	–	100	100	90	27	53
Control	–	–	–	0	–	–

[a] 15 to 25 cotton bollworms per test were used.
[b] Assay was made on cells plus supernatant fluids.

TABLE 3. The neutralizing effect of antiserum on *Heliothis zea* nuclear polyhedrosis virus as tested in neonate cotton bollworms

Treatment	No. dead/total	% mortality
Virus[a]	8/8	100
Virus + preantiserum	8/8	100
Virus + postantiserum	0/8	0
Virus + postantiserum (1:2)	0/8	0
Virus + postantiserum (1:4)	0/7	0
Virus + postantiserum (1:8)	3/7	43
PIB[b] + postantiserum	7/7	100
Control (uninoculated)	0/10	0

[a] 0.6 ml of virus inoculum (released by digestion of 10^9 PIB) per test was employed.
[b] 0.6 ml of 10^5 PIB/ml per test was used.

mortality rate for fluids and cells from WI38 at this period is unknown. No attempt was made to titrate the virus.

Immunofluorescence. Since virus could be recovered from inoculated PHA cultures after 4 weeks, it was of interest to determine whether or not antigen could be detected in such cultures since it is possible for viral coded antigens to be synthesized without viral multiplication (Tevethia *et al.*, 1965). Coverslips were removed both from controls and from inoculated cultures at weekly intervals for 5 weeks. No foci of fluorescence were observed in inoculated or control cultures.

Neutralization test. In order to assess the potency of the antiserum prepared against the NPV of *H. zea*, a neutralization test was developed. As can be seen from Table 3, postantiserum completely neutralized the virus up to a 1:4 dilution of antiserum, whereas undiluted preantiserum failed to do so. Of interest also was the failure of postantiserum to neutralize PIB. This experiment was repeated with similar results.

DISCUSSION

Inoculation of human cell cultures with PIB and free virions of *Heliothis* NPV had no adverse effect on cell viability; neither was any CPE produced in such cultures. Furthermore, no accumulation of viral antigen in inoculated PHA cells as determined by immunofluorescence could be demonstrated over a 5-week period. *Heliothis* NPV did not transform either PHA or LEU cultures. However, in one out of three experiments *Heliothis* NPV did enhance the transformation of PHA cultures by SV40. The most likely possibilities which might explain this lack of reproducibility include differences in the genetic constitution of amnion cells which were derived from three different placentas and the presence of adventitious agents in the PHA cultures in which enhancement was demonstrated. Further studies are being conducted

with *Heliothis* NPV and other insect viruses in an attempt to elucidate this phenomenon.

The recovery of *Heliothis* NPV from mammalian cell cultures 4 weeks after inoculation represents the first report concerning the persistence of an insect virus in vertebrate cell cultures. Since culture fluids were changed at least once a week, it is unlikely that recovery of virus was due solely to extracellular virus remaining from the inoculum. Although it has been reported that *Heliothis* NPV retains approximately 50% of its infectivity after 14 days at 37 C in cell-free diluent (Ignoffo and Rafajko, 1972; Shapiro and Ignoffo, 1969), no prior information concerning the infectivity of this virus from inoculated mammalian cultures has been reported. Electron microscopic studies are in progress to determine if actual penetration of cells by the virus occurred.

The development of a neutralization test for *Heliothis* NPV whereby the potency of antiserum can be assessed should be applicable also to other insect viruses. The titer of the antiserum in this study, although low, could possibly be increased by employing a more severe course of immunization. Since it is the virions contained in the PIB which are infective, the infectivity of released virions from PIB treated with antiserum is not surprising.

These results further support the findings of Ignoffo and Rafajko (1972) concerning the nonlethal effect of *Heliothis* NPV for primate cells. However, further studies should be made before other insect viruses are employed on a large scale for biological control. The finding by Longworth *et al.* (1973) that domestic and wild animals have IgM antibodies that react with insect viruses raises some very interesting questions with regard to possible infection of higher species with these types of viruses.

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